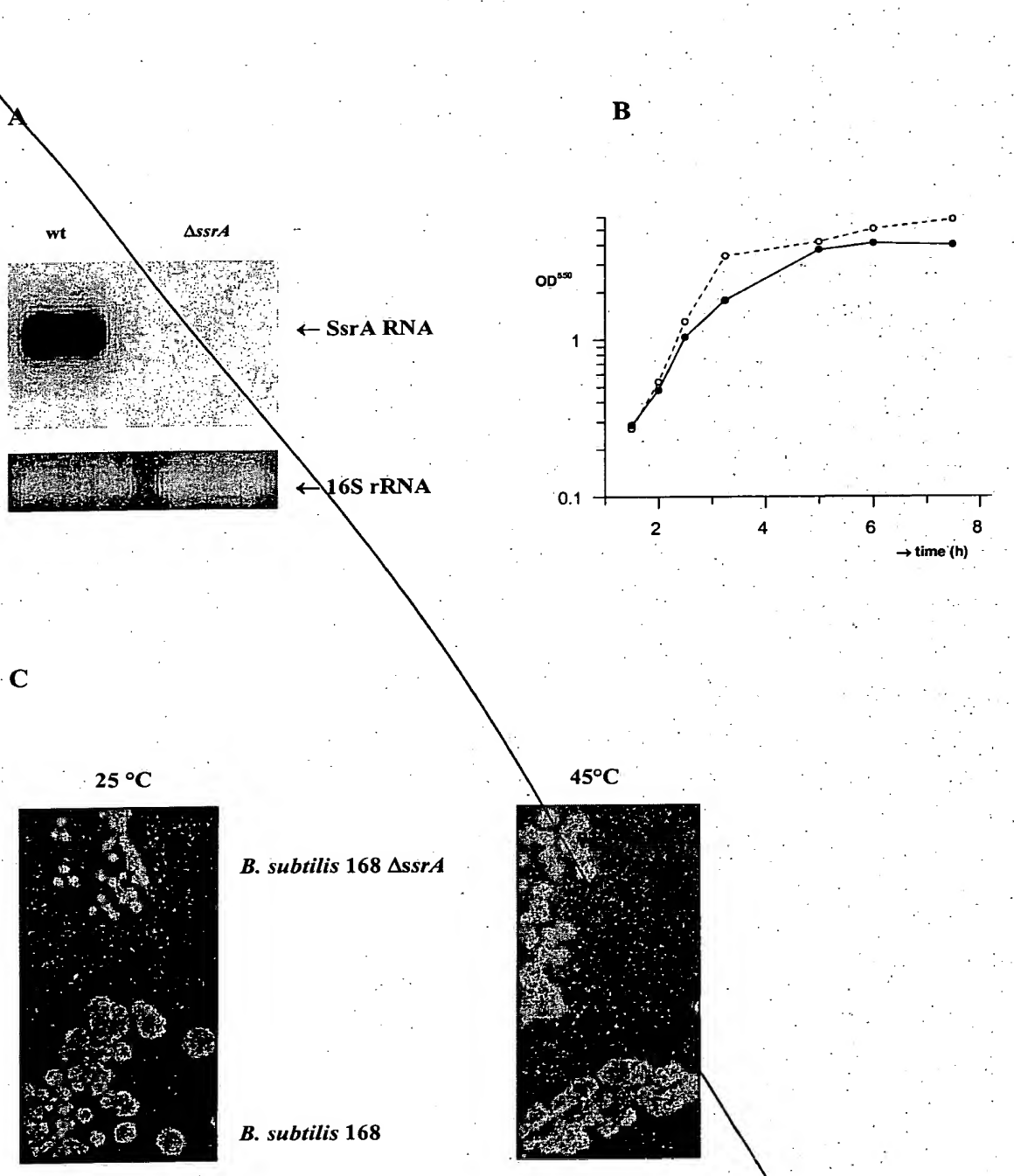


FIG. 1. A. Northern blot of total RNA of *B. subtilis* 168 and *B. subtilis* 168 Δ*ssrA*, hybridized with an *ssrA* specific probe. At the bottom: the level of 16S RNA in both RNA samples. B. Growth curves of *B. subtilis* 168 (---o---) and *B. subtilis* 168 Δ*ssrA* (—●—) at 37 °C in TSB medium. C. Growth of *B. subtilis* 168 and *B. subtilis* 168 Δ*ssrA* on HI-agar plates at 25 °C or 45 °C.

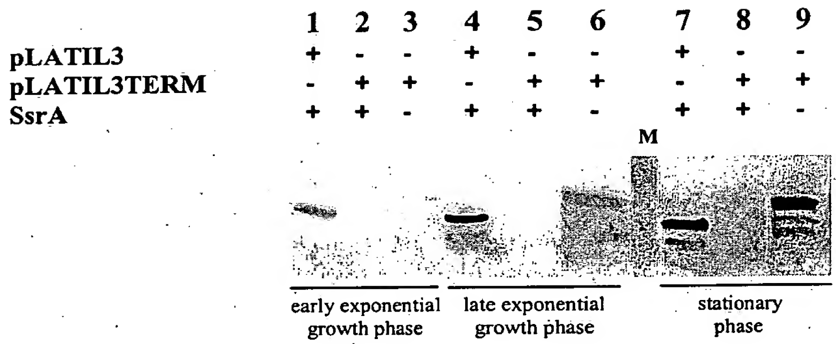
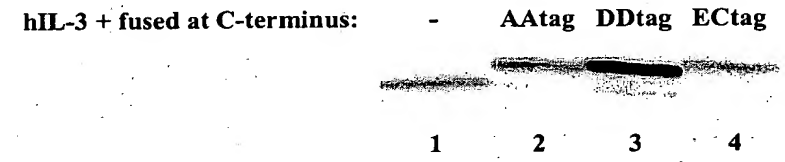


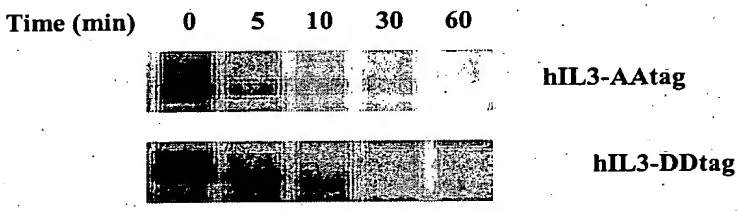
FIG. 2. hIL-3 expressed from an mRNA without a stop codon (pLATIL3TERM), accumulates in the medium of *B. subtilis* lacking SsrA (lanes 3, 6, 9), but not in cells containing functional SsrA (lanes 2, 5, 8). At three different growth stages, samples were collected from cultures of *B. subtilis* 168 (pLATIL3) [lanes 1, 4, 7], *B. subtilis* 168 (pLATIL3TERM) [lanes 2, 5, 8], and *B. subtilis* 168 Δ*ssrA* (pLATIL3TERM) [lane 3, 6, 9]. After centrifugation, the proteins in the culture supernatants were concentrated by TCA precipitation and analyzed by SDS-PAGE and Western blotting with anti-hIL-3 antibodies. The amount of total extracellular protein of *B. subtilis* 168 (pLATIL3) that was applied to the gel [lanes 1, 4, 7] was 10 times less then that of *B. subtilis* 168 (pLATIL3TERM) [lanes 2, 5, 8] or *B. subtilis* 168 Δ*ssrA* (pLATIL3TERM) [lanes 3, 6, 9]. M indicates a lane with a prestained protein ladder; the molecular weight of the upper band corresponds to 20 kDa, that of the lower band to15 kDa.

FIG. 3. Stability of hIL-3 variants with different C-terminal tags.
(A). Western blot analysis of hIL-3 protein variants produced by *B. subtilis* 168 transformed with plasmid pLATIL3 (lane 1), pLATIL3BStag (expression of hIL-3 with a C-terminal *B. subtilis* SsrA tag (AA-tag): hIL-3-AGKTNSFNQNVALAA; lane 2), pLATIL3DDtag (expression of hIL-3 with a DD-tag: hIL-3-AGKTNSFNQNVAL**DD**; lane 3), and pLATIL3ECtag (expression of hIL-3 with a C-terminal *E. coli* SsrA tag (EC-tag): hIL-3-AANDENYALAA; lane 4). Culture supernatants of cells entering the stationary phase were collected and analyzed by SDS-PAGE and Western blotting with anti-hIL-3 antibody.
(B). Pulse-chase assays: Cells of *B. subtilis* 168 (pLATIL3BStag) and 168 (pLATIL3DDtag) were labeled with [^{35}S]-methionine for 1' prior to chase with excess non-radioactive methionine. Samples were withdrawn at the times indicated, centrifuged and the culture supernatants were analyzed by SDS-PAGE and fluorography.
(C). The amounts of hIL-3-AAtag and hIL3-DDtag in (B) were quantified by determination of the radioactivity in the dried gel using a PhosphorImager (Molecular Dynamics) and plotted.

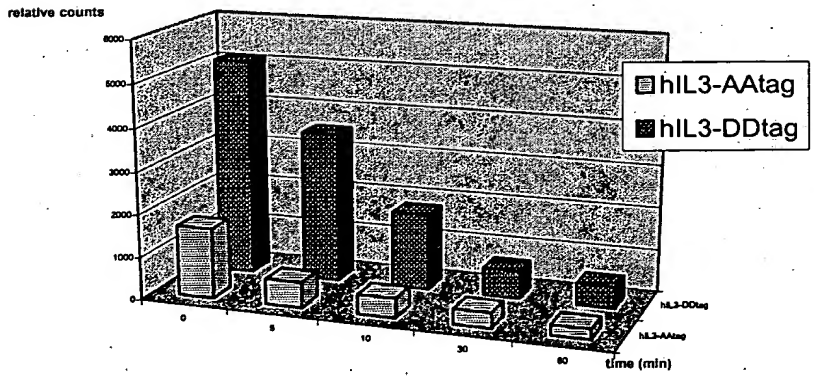
A



B



C



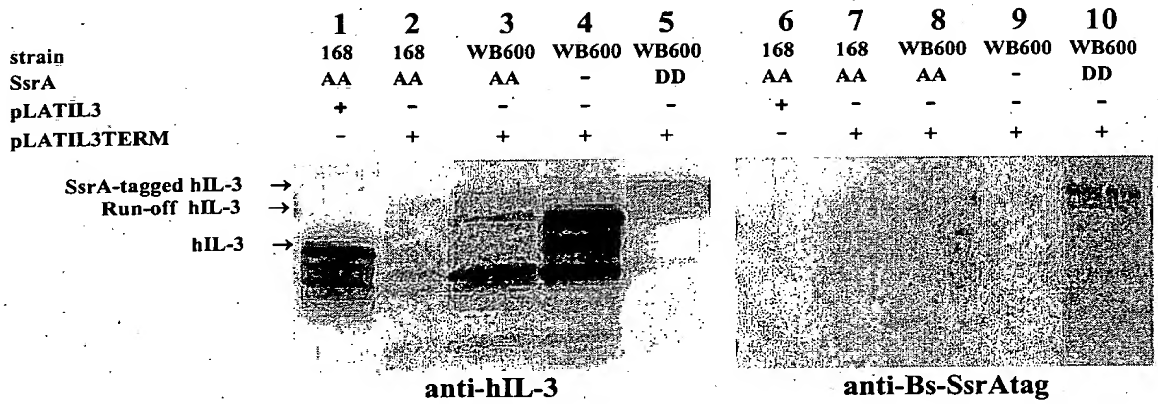


FIG. 4. The 'major extracellular proteases' of *B. subtilis* play a role in the degradation of extracellular, SsrA-tagged h-IL3. Western blot analysis of hIL-3 protein secreted by *B. subtilis* 168 harboring plasmid pLATIL3 (lane 1, 6) or pLATIL3TERM (lane 2, 7), and *B. subtilis* WB600 (a multiple protease negative strain) containing plasmid pLATIL3TERM and expressing either wild-type SsrA (lane 3, 8), no SsrA (lane 4, 9) or $SsrA^{DD}$ (lane 5, 10). Culture supernatants of cells entering the stationary phase were collected, concentrated by TCA precipitation, analyzed by SDS-PAGE and immunoblotting with anti-hIL-3 antibody (lanes 1-5) or anti-Bs-SsrAtag antibody (lanes 6-10). SsrA-tagged hIL-3 (lanes 3, 5, 8, 10), run-off hIL-3 translation product (lane 4, and possibly also in lane 3 and 5, see text), and wild-type hIL-3 (lane 1) are indicated by the arrows (→). Protein bands with lower molecular weight that also react with anti-hIL-3 antibody are supposedly degradation products of hIL-3, SsrA-tagged hIL-3 or run-off hIL-3 translation product.

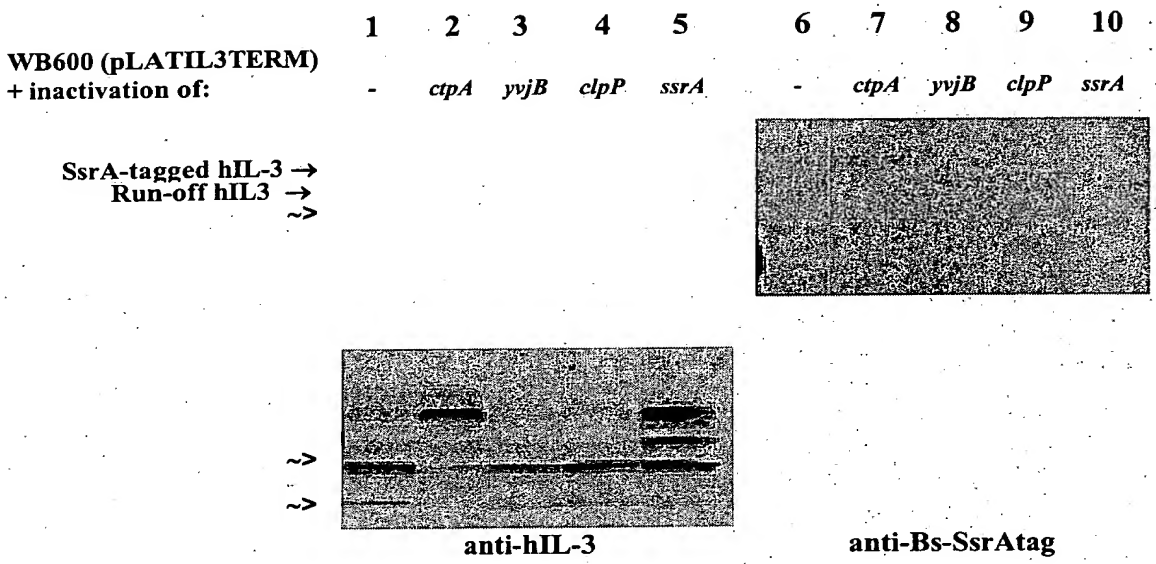


FIG. 5. *B. subtilis* CtpA has an additional role in the degradation of SsrA-tagged hIL-3. Western blot analysis of hIL-3 protein secreted by *B. subtilis* WB600 harboring plasmid (pLATIL3TERM) and carrying either no additional mutation (lane 1, 6), or lacking CtpA (lane 2, 7), YvjB (lane 3, 8), ClpP (lane 4, 9), or SsrA (lane 5, 10). Culture supernatants of cells entering the stationary phase were collected, concentrated by TCA precipitation, analyzed by SDS-PAGE and Western blotting with anti-hIL-3 antibody (lane 1-5) or anti-Bs-SsrAtag antibody (lane 6-10). The straight arrows (→) mark SsrA-tagged hIL-3 (lanes 1-4 and lanes 6-9), and run-off translation product (lane 5 and possibly (see text) also in lanes 1-4). Degradation products of (SsrA-tagged) hIL-3 are indicated by ~>.

FIG. 6.

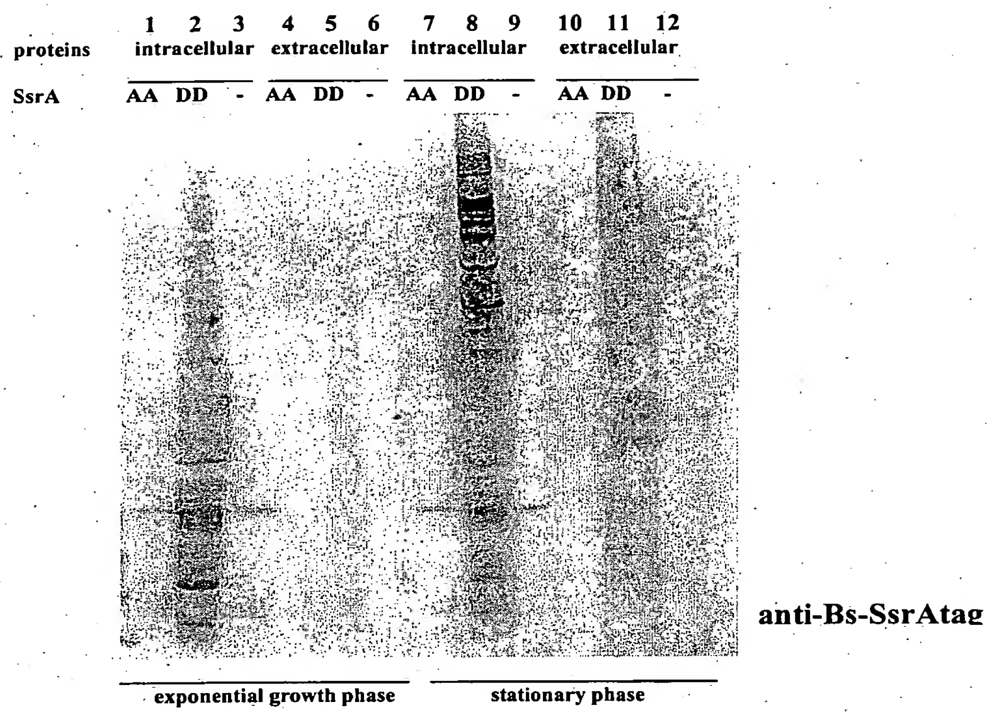


FIG. 6. Tagging of native *B. subtilis* proteins. Total intracellular or extracellular proteins produced by cells in the exponential growth phase or stationary phase of *B. subtilis* 168 expressing wild-type SsrA (AA), 168 I*ssrA*^{DD} expressing SsrA^{DD} (DD), or 168 Δ*ssrA* containing no SsrA RNA (-) were analyzed by Western blotting using anti-Bs-SsrAtag antibody.

Figure 7

IL-3 amino acid sequence (Native sequence associated with its native signal sequence)

MSRLPVLLLL QLLVRPGLQA PMTQTTPLKT SWVNCSNMID EIITHLKQPP
LPLLDFNNLN GEDQDILMEN NLRRPNLEAF NRAVKSLQNA SAIESILKNL
LPCLPLATAA PTRHPIHIKD GDWNEFRRKL TFYLKTLENA QAQQTTLSLA IF

IL-3 as encoded by plasmid pLATIL3

MKQQKRLYAR LLTLLFALIF LLPHSSASAA PMTQTTPLKT SWVNCSNMID
EIITHLKQPP LPLLDFNNLN GEDQDILMEN NLRRPNLEAF NRAVKSLQNA
SAIESILKNL LPCLPLATAA PTRHPIHIKD GDWNEFRRKL TFYLKTLENA
QAQQTTLS

IL-3 amino acid sequence (Substituted tag charged C-terminus)

MKQQKRLYAR LLTLLFALIF LLPHSSASAA PMTQTTPLKT SWVNCSNMID
EIITHLKQPP LPLLDFNNLN GEDQDILMEN NLRRPNLEAF NRAVKSLQNA
SAIESILKNL LPCLPLATAA PTRHPIHIKD GDWNEFRRKL TFYLKTLENA
QAQQTT*DD*

IL-3 amino acid sequence (Tagged[14])

MKQQKRLYAR LLTLLFALIF LLPHSSASAA PMTQTTPLKT SWVNCSNMID
EIITHLKQPP LPLLDFNNLN GEDQDILMEN NLRRPNLEAF NRAVKSLQNA
SAIESILKNL LPCLPLATAA PTRHPIHIKD GDWNEFRRKL TFYLKTLENA
QAQQTTLS*AG KTNSFNQNVA LDD*